Acta Physiol Scand 2001, 171, 9–16

Nitrite-derived nitric oxide: a possible mediator of 'acidic–metabolic' vasodilation

A. MODIN,[1,2] H. BJÖRNE,[3] M. HERULF,[1] K. ALVING,[1] E. WEITZBERG[3] and J.O.N. LUNDBERG[1]

1 Department of Physiology and Pharmacology, Karolinska Institute, Stockholm, Sweden
2 Department of Clinical Physiology, Karolinska Hospital, Stockholm, Sweden
3 Department of Anaesthesiology and Intensive Care, Karolinska Hospital, Stockholm, Sweden

ABSTRACT

The fundamental, yet poorly understood, physiological mechanism known as 'acidic–metabolic' vasodilation, contributes to local blood flow regulation during hypoxia/ischaemia and increased metabolic activity. The vasodilator nitric oxide (NO) has been suggested to be involved in this event. Besides enzymatic production by NO synthases, a novel mechanism for generation of this gas *in vivo* was recently described. This involves non-enzymatic reduction of inorganic nitrite to NO, a reaction that takes place predominantly during acidic/reducing conditions. We have studied the effects of physiological amounts of nitrite on NO generation and relaxation of rat aorta *in vitro* in a situation where environmental pH was reduced to levels seen in tissues during hypoxia/ischaemia. The relaxatory effect of nitrite was increased in an acidic buffer solution (pH 6.6) compared with neutral pH; EC50 for nitrite was reduced from 200 to 40 μM. Nitrite-evoked relaxation was effectively prevented by coadministration of an inhibitor of soluble guanylyl cyclase. The relaxation was further potentiated by the addition of ascorbic acid. In parallel, NO was generated from nitrite in a pH dependent manner with even larger amounts seen after addition of ascorbic acid. NO generation from nitrite correlated to the the degree of relaxation of rat aorta. These results illustrate non-enzymatic release of NO from nitrite at physiological concentrations. This may be an important auto-regulated physiological mechanism involved in the regulation of vascular tone during hypoxia/ischaemia.

Keywords acidosis, blood flow, low pH, nitric oxide, nitrite, ODQ, vascular smooth muscle, vasodilation.

Received 10 May 2000, accepted 30 August 2000

For more than a century, it has been known that acidic solutions evoke vascular smooth muscle relaxation (Gaskell 1880) and more recent studies have shown that reduction of blood pH increases blood flow (Zsotér *et al.* 1961). This phenomenon, the so-called 'acidic–metabolic vasodilation', is suggested to contribute to the regulation of local blood flow, the vasodilation which occurs during hypoxia or ischaemia or during increased metabolic activity in order to fulfil the need for energy and oxygen supply (see Guyton & Hall 1996). In line with the involvement of multiple events behind this local vasodilation and the observation that the blood flow regulation varies between various types of vascular beds, several factors and mechanisms, besides the hydrogen ion concentration, have been suggested to be involved. Prostanoids, purines and transmitters from sensory nerves are likely candidates as specific factors (see Franco-Cereceda *et al.* 1993, Aalkjaer & Poston 1996, Ishizaka & Kuo 1996) but also the more recently verified vasoactive molecule nitric oxide (NO) may interact (see Aalkjaer & Poston 1996). It has been shown that NO release from rat and guinea-pig heart is increased during hypoxia (Zweier *et al.* 1995, Giannella *et al.* 1997). Nitric oxide is produced from L-arginine and molecular oxygen by specific enzymes, NO synthases (NOS) (Mayer *et al.* 1989). In addition to its major importance in blood flow regulation (Furchgott & Zawadzki 1980, Palmer *et al.* 1987, Ignarro *et al.* 1987) NO takes part in host-defence reactions (Nathan & Hibbs 1991) and is involved in nerve transmission (Gillespie *et al.* 1989). In the cardiovascular system, large amounts of NO are produced from the

Correspondence: J. O. N. Lundberg, Division of Pharmacology, Department of Physiology and Pharmacology, Karolinska Institute, S-171 77 Stockholm, Sweden. Email: jon.lundberg@fyfa.ki.se

endothelium in response to endothelium-dependent vasoactive substances (e.g. acetylcholine, substance P) or to shear stress (see Moncada *et al.* 1991). The most common way to study actions of NO has been to interfere with the enzymatic production of this gas and the same experimental approach has been used in studies of involvement of NO in metabolic vasodilation (You *et al.* 1994, Aalkjaer & Poston 1996). However, recently an endogenous formation of NO, independent of NOS was described (see Weitzberg & Lundberg 1998). This non-enzymatic NO formation has been shown to occur in humans in the stomach (Lundberg *et al.* 1994), in the oral cavity (Duncan *et al.* 1995), in urine (Lundberg *et al.* 1997) and in the ischaemic rat heart (Zweier *et al.* 1995) and is related to the reduction of nitrite (NO_2^-) in an acidic/reducing environment (see Weitzberg & Lundberg 1998). NO formation in the stomach is pH dependent and results from acidification of nitrite present in swallowed saliva (Lundberg *et al.* 1994). Conversely, nitrite is a metabolite of NO and is present throughout the body in the μM range in fluids and tissues (Friedberg *et al.* 1997). Nitrites have long been known to act as vasodilating substances (see Goodman & Gilman 1941). It is a well-known phenomenon that nitrite, when acidified, dilates vessels through the release of NO (Furchgott *et al.* 1987). However, the concentration of nitrite and acidity (~pH 2) in earlier experiments have been far from physiologic conditions. The aim of the present study was to investigate whether nitrite in physiologically relevant concentrations, upon reaching a physiologically acidic milieu (~pH 6.6), would act as a vasodilator through NO-release. Thus we wanted to find out whether non-enzymatically derived NO, like NOS-derived NO, may be involved in the regulation of vascular tone.

METHODS

Functional experiments – organ bath

Wistar rats of 150–250 g body weight were killed with an overdose of pentobarbitone given intraperitoneally and the aorta was rapidly and carefully dissected out and placed in cold modified Krebs buffer solution (pH 7.45). The aorta was cut in circular segments (1–2 mm length) and mounted on two thin metal holders, one connected to a force displacement transducer (Grass model FTO3) and the other to a movable device in an organ bath. The movable device allowed application of a passive tension of 5 mN (see Högestätt *et al.* 1983) on the aorta segment and tension was recorded on a Grass polygraph (model 7B). The organ bath was 2 mL and it was filled with buffer solution (pH 7.45) and bubbled with 6.5% CO_2 in O_2. The temperature of the buffer was kept constant at 37.5 °C.

The aortic segments were allowed to equilibrate for 60 min, before their contractility was tested by application of KCl (40 mM) and phenylephrine (phe, 1 μM). Only vascular segments giving responses to both these constricting agents were used for the study. After washout and in some experiments after a change to low pH buffer (around 6.6), phe was readministered and the vessels developed a stable contraction approximately 20–30 min later. In both neutral and low pH buffer, the phe-evoked contraction lasted for more than 60 min if no additional drug was added and the degree of contraction was similar. Changing the buffer in the organ bath from neutral to acidic buffer did not evoke any changes of the baseline vascular tone. Following phe-evoked contraction, doses of sodium nitrite (0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000 μM) were added in a cumulative order to organ baths containing neutral or acidic buffer solution, alone, or in combination with 0.12 M HCl or ascorbic acid (0.5 mM). When sodium nitrite was combined with ascorbic acid or HCl, sodium nitrite was first added to the organ bath and then, within a few seconds, the other substance was added. Sodium nitrite was also administered to pre-contracted aortic segments which had been incubated with the inhibitor of soluble guanylyl cyclase [1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) (Garthwaite *et al.* 1995) (10 μM, administered together with phe) in the presence of low pH buffer solution. Corresponding control segments were run in parallel experiments. Relaxatory effects of the NO-donor *S*-nitroso-*N*-acetylpenicillamine (SNAP, 0.0001, 0.001, 0.01, 0.1, 1 μM) were also tested on phe-contracted aortic segments. In one group of rats SNAP was added cumulatively in increasing concentrations in neutral buffer solution and in a separate group of experiments, SNAP was added to aortic segments in neutral or acidic buffer solution. In order to exclude influence of NO derived from nitric oxide synthase, all experiments were performed in the presence of the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) (100 μM), which was added to the organ bath simultaneously with phe. In separate experiments 100 μM L-NAME was shown to totally block the vasorelaxing effects of 1 μM acetylcholine. The volume of application of the drugs to the organ bath was 50 μL or less and the pH in the organ bath was measured repeatedly (Beckman, Futura™).

Measurement of NO

In order to detect NO formation from sodium nitrite in a situation similar to the functional experiments above, a closed chamber, a canister, with a total volume of 580 mL was used as an artificial organ bath. The canister was filled with the same solutions and substances as had been used in the functional experi-

ments, i.e. neutral or acidic buffer solution (200 mL), L-NAME (100 μM, 5 mL) and phe (1 μM, 5 mL) and after closed incubation for 5 min at room temperature, the content of NO in the head space gas above the buffer was immediately analysed with a chemiluminescence analyser (CLD 700, Eco Physics, Dürnten, Switzerland). The detection limit for NO was 1 part per billion (ppb) and the analyser was calibrated at known concentrations of NO in nitrogen, by an electromagnetic flow controller (Environics, Middletown, CT, USA). Sodium nitrite, alone or in combination with ascorbic acid (0.5 mM, 5 mL) or 0.12 M HCl (0.6–1.2 mL), was added in increasing concentrations to the chamber. As in the organ baths, sodium nitrite was first added and then ascorbic acid or HCl was added within a few seconds. After 5 min incubation, the monitored peak level of NO was noted for each nitrite concentration. The pH of the buffer was measured at the beginning and at the end of the experiment. The NO concentration in the headspace was also measured after addition of SNAP to neutral buffer containing L-NAME (100 μM) and phe (1 μM). SNAP was added in an increasing cumulative manner in concentrations of 0.0001, 0.001, 0.01, 0.1 and 1 μM and each dose was incubated for 5 min at room temperature before NO was measured.

Buffer solutions and drugs

The buffer solutions contained (mM): KCl 4.8, $MgSO_4$ 1.15, $CaCl_2$ 2.5 and glucose 11.1. The buffer adjusted to a pH of 7.45 also contained (mM): NaCl 118, $NaHCO_3$ 25 and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethansulphonic acid (HEPES) 20, and the buffer adjusted to a pH of 6.6 also contained (mM): NaCl 141, $NaHCO_3$ 2 and (2-[N-morpholino]ethanesulphonic acid) (MES) 20. The final adjustment of pH was made after 30 min of equilibration with the carbogen gas (6.5% CO_2 in O_2) by adding appropriate amounts of 3 M NaOH.

1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) was purchased from Tocris Cookson (Bristol, UK) and was first dissolved in dimethyl sulphoxide (DMSO) and further diluted in distilled water. SNAP (SNAP, Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) was first dissolved in ethanol and further diluted in distilled water. All other drugs were dissolved in distilled water. L-NAME and phe were from Sigma (St Louis, MO, USA) and ascorbic acid was from Apoteksbolaget (Göteborg, Sweden).

Calculations

The vasodilatory effects were calculated as percentage of the maximal precontracted tone. Data are given as mean ± SEM of number of aortic segments (n) and the number of rats is indicated as (N). The EC50 value for each drug was here defined as the concentration which produced 50% relaxation of phe-contracted level under each respective condition. The threshold dose was defined as the lowest concentration of the drug used which evoked effects in more than 60% of the preparations studied. Statistical analyses are performed on (n) using Mann–Whitney test for unpaired observations and Spearman's rank-test for linear correlation using the statistical computer program Instat 2.01.

RESULTS

Functional experiments – organ bath

Sodium nitrite evoked a dose-dependent relaxation of the phe-precontracted aorta segments (Figs 1 and 2). In the neutral buffer solution, the threshold dose of sodium nitrite was 10 μM, the EC50 value 200 μM and the aortic segment relaxed almost to basal tone following the highest dose of 1000 μM nitrite. In the presence of the acidic buffer, the threshold dose of nitrite as well as the EC50 value were reduced to 2.5 and 40 μM, respectively, giving a leftward-shifted dose–response curve. At a cumulative dose of 44 μM nitrite, the aortic segment was relaxed by 12 ± 2% in neutral buffer compared with 53 ± 8% at low pH ($P < 0.001$, Fig. 2).

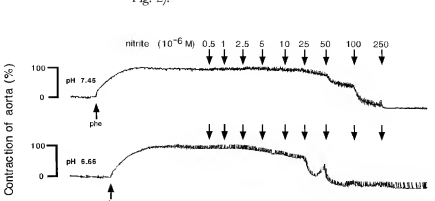

Figure 1 Original recordings of isolated segments of rat aorta in organ bath containing modified Krebs solution of neutral pH (upper curve) and low pH (lower curve) when contracted with phe (10^{-6} M) followed by relaxation evoked by an increasing concentration of sodium nitrite. Time scale is indicated for 60 s.

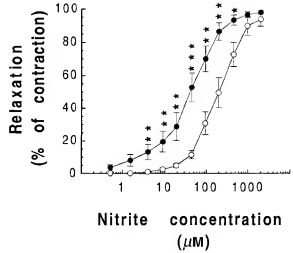

Figure 2 Concentration-response curves showing the relaxatory effects of sodium nitrite, added cumulatively, on isolated segments of rat aorta contracted with phe (10^{-6} M). The organ bath contained modified Krebs solution of neutral pH (○, $n = 14$, $N = 6$) or of low pH (●, $n = 14$, $N = 7$). Significant differences between the relaxatory effects at neutral compared with low pH buffer solution are indicated, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

In a separate group of phe-contracted segments of rat aorta, ascorbic acid was added together with sodium nitrite and the dose response curves were shifted to the left both in the presence of neutral and of acidic buffer (Fig. 3) if compared with the situation without ascorbic acid (Fig. 2). However, ascorbic acid per se reduced the pH in the organ bath, 0.12 M HCl was co-administered with sodium nitrite in control experiments in order to evoke similar pH reduction. Addition of ascorbic acid, or 0.12 M HCl, reduced the pH by approximately 0.02 pH units per dose and the pH in the ascorbic acid group and the HCl group did not significantly differ from each other. Thus, at similar pH in the organ baths, the dose response curve of sodium nitrite-evoked relaxation was significantly leftward-shifted by ascorbic acid compared with the HCl group (Fig. 3).

In the presence of ODQ (at low pH buffer solution), the dose–response curve for sodium nitrite evoked relaxation of the phe-contracted aorta was rightward shifted (Fig. 4). The threshold dose for relaxatory effects was 500 μM, to be compared with 10 μM in the absence of ODQ (presence of vehicle). Furthermore, when the concentration of sodium nitrite was increased to 10 000 μM, the maximal relaxation was 50 ± 8% of the precontracted level. There were no differences between the effects in the presence of the vehicle for ODQ compared with control (i.e. in low pH buffer solely).

In the neutral buffer solution, SNAP evoked relaxations of phe-contracted aortic segments in a concentration-dependent manner. Following a SNAP concentration of 0.01 μM the vessels had relaxed by 18 ± 8% and at 1 μM all segments were completely relaxed, i.e. had returned to baseline. The vehicle for SNAP, containing increasing amounts of ethanol, did not evoke any vascular effects. Furthermore, in a separate group of experiments ($n = 6$–8, $N = 4$, not shown), the relaxatory effect of SNAP on phe-contracted aortic segments in low-pH buffer solution was slightly more pronounced compared with the neutral buffer solution. The vasorelaxation evoked by 0.0001, 0.001, 0.01 and 0.1 μM SNAP was approximately 10% more pronounced in the acidic buffer solution.

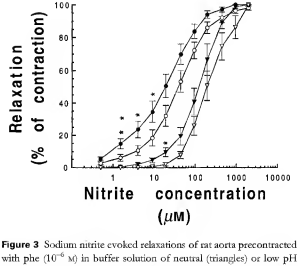

Figure 3 Sodium nitrite evoked relaxations of rat aorta precontracted with phe (10^{-6} M) in buffer solution of neutral (triangles) or low pH (circles). Sodium nitrite was added in a cumulative manner and in combination with 0.5 mM ascorbic acid (solid symbols) or 0.12 M HCl (open symbols). Significant differences between the relaxatory effects in the presence of ascorbic acid compared with in the presence of HCl in the respective buffers are indicated, $^{*}P < 0.05$, $^{**}P < 0.01$ ($n = 7$–18, $N = 5$–7).

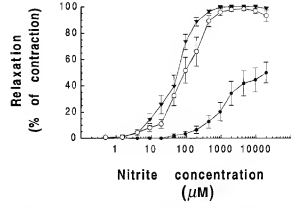

Figure 4 Relaxation of isolated segments of rat aorta, precontracted with phe (10^{-6} M), by sodium nitrite added in a cumulative manner. The organ bath contained modified buffer solution of low pH and sodium nitrite was added in the presence of ODQ (10^{-5} M, ● $n = 15$, $N = 4$), vehicle (▼$n = 11$, $N = 3$) or solely buffer solution (control ○, $n = 16$, $N = 3$).

Measurement of NO

The amount of NO detected in the artificial organ bath increased in parallel with increased amounts of added sodium nitrite (Fig. 5). In control conditions, the NO level increased from 2.5 ± 0.8 (in the absence of sodium nitrite) to 8.6 ± 1.3 ppb following the dose of 1000 μM sodium nitrite. In acidic buffer solution, the corresponding levels were 2.5 ± 0.78 and 37 ± 13 ppb. Addition of ascorbic acid or 0.12 M HCl in combination with each dose of sodium nitrite increased the NO formation further. In parallel, the pH values in the artificial organ baths were reduced. Combination of sodium nitrite and ascorbic acid evoked a maximal NO level of 1141 ± 170 ppb, to be compared with 178 ± 46 ppb in the presence of HCl, detected 5 min after the dose of 1000 μM sodium nitrite. Furthermore, 5 μM sodium nitrite in the presence of ascorbic acid evoked an NO level of 12 ppb, which was close to the levels evoked by 100 μM sodium nitrite in the acidic buffer alone or by 50 μM sodium nitrite with addition of 0.12 M HCl.

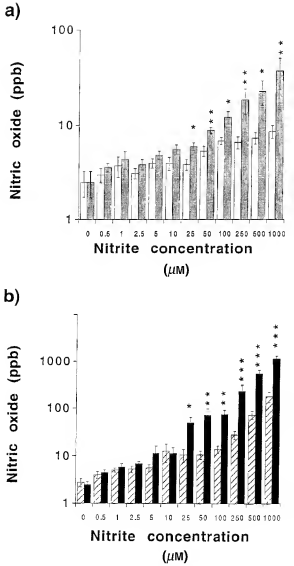

Figure 5 Nitric oxide levels, ppb, measured in a canister containing buffer solution, phe (10^{-6} M) and L-NAME (10^{-4} M) after addition of sodium nitrite in a cumulative manner. Levels of NO are measured in presence of neutral buffer solution (□) or in low pH buffer solution (▨) (a), and in low pH buffer solution with addition of 0.12 M HCl (▩) or in low pH buffer solution with addition of 0.5 mM ascorbic acid (■) (b). Significant differences between the levels in the respective figures are indicated, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ ($n = 6$–8).

The concentration of NO in the artificial organ bath was also measured following the addition of increasing concentrations of SNAP. When 1 μM of SNAP was added, the NO level was 14 ± 2.3 ppb, to be compared with 5.2 ± 1.2 in the absence of SNAP ($P < 0.05$). Comparison of the SNAP-evoked NO levels in the artificial organ bath with the SNAP-evoked relaxatory effects on precontracted aortic segments evoked by same concentrations of SNAP, showed a significant correlation ($r = 0.93$, $P < 0.05$). The levels of NO detected at the specific concentrations of sodium nitrite were also correlated to the vasorelaxatory effects under comparable conditions (neutral or acidic environment including presence of HCl or ascorbic acid) and the correlation was significant ($r = 0.81$, $P < 0.0001$).

DISCUSSION

The present study demonstrates that physiological levels of nitrite induce vasodilation of rat aorta *in vitro* when the surrounding pH is reduced from 7.45 to 6.6. This nitrite-evoked relaxatory effect was inhibited by the guanylyl cyclase inhibitor ODQ. Moreover, NO levels detected in the head space gas above the acidic buffer solution containing nitrite, were higher than the levels detected above the neutral buffer solution. These results illustrate that nitrite is more potent as a vasodilator if the environment is acidified, that the vasorelaxation is cGMP-mediated and suggest that nitrite exerts its vasodilatory effect via generation of NO. The fact that acidic nitrite releases NO is well known and acidic nitrite has been used as an NO-donating drug in studies of EDRF/NO (Furchgott *et al.* 1987). However, in earlier studies experimental nitrite concentrations as well as pH have been far outside normal physiological ranges. The importance of the present results is that when both nitrite concentration and pH values are kept within the normal physiological range, vasodilation occurs with parallel release of NO. It has previously been reported that endogenous nitrite converts to NO in acidic environment such as in the stomach (Lundberg *et al.* 1994), but in the stomach the pH is approximately 2, and in the present study, it is

demonstrated that even at a much higher pH (~6.6) there is a clear-cut augmentation of NO conversion from nitrite. Nitrite is present throughout the organism but the specific nitrite concentration varies depending on the tissues and the species investigated and on methods used. For example, lung tissues have shown levels of about 500 μM in rats (Deshmukh *et al.* 1997) and 152 μM in humans (Friedberg *et al.* 1997); nitrite concentration is 69 μM in human kidney (Friedberg *et al.* 1997) and 12 μM in rat heart (Zweier *et al.* 1995). In plasma, the levels reported are generally lower than those in tissue: 83 μM in rat plasma (Deshmukh *et al.* 1997), 0.45 μM in human plasma (Leone *et al.* 1994), 6.6 μM in human serum (Friedberg *et al.* 1997). In plasma, nitrite is quickly oxidized to nitrate following reaction with haemoglobin, and therefore it is possible that tissue-bound nitrite represents the major source of non-enzymatic NO. The reported tissue levels of nitrite are clearly in the range where major vasorelaxation was observed. In rat hearts, it has been demonstrated that both in the presence and absence of L-NAME, ischaemia increases the NO production (Zweier *et al.* 1995), results which support the present suggestion of an involvement of non-enzymatically derived NO in vascular smooth muscle relaxation under acidic conditions.

The acidic environment in the present *in vitro* set up was represented by a buffer solution with a pH of approximately 6.6. The reason for this more acidic condition compared with that used in other studies of acidic vasodilation (see Aalkjaer & Poston 1996), was to obtain a clear difference compared with neutral pH and at the same time remaining within the physiological range. For comparison, the pH in muscle during heavy physical work may be as low as 6.4–6.5 (Victor *et al.* 1988, Nishiyasu *et al.* 1994). In the rat heart subjected to approximately 3 min of ischaemia, the pH was 6.7 and following 10 min ischaemia a pH of 5.9 was measured (Zweier *et al.* 1995).

The formation of NO from acidified nitrite is greatly enhanced in the presence of vitamin C (Bartsch *et al.* 1988, Lundberg *et al.* 1997). Furthermore, ascorbic acid has been shown to effectively scavenge superoxide, thereby preserving NO (see Jackson *et al.* 1998). In a post-ischaemic situation, the formation of oxygen free radicals is profound (see, e.g. Bonne *et al.* 1998, Ferrari *et al.* 1998). When comparing the effects in baths with ascorbate, with baths containing HCl at the same pH, it was shown that ascorbic acid had an intrinsic capability of increasing NO generation from nitrite, independent of pH. This illustrates another catalyser for nitrite conversion to NO and strengthens the view of NO being the mediator of nitrite-induced vasodilation. Jackson *et al.* (1998) showed that 0.5 mM ascorbic acid, that is, the same concentration used in the present study, scavenges superoxide. The reason for repeated administration of ascorbic acid was that oxidation of ascorbic acid occurs continuously. The concentrations of ascorbic acid used in the present study were higher than the reported concentrations of ascorbic acid in human plasma (about 0.05 mM) (Levine *et al.* 1996), but lower than what has been reported intracellularly (about 2 mM) (see Jackson *et al.* 1998). Thus, one might speculate that, for example, in skeletal muscle during heavy exercise, the pH-reduction may stimulate non-enzymatic formation of NO, especially if high concentrations of both nitrite and ascorbate are present.

The acidic vasodilation has mainly been suggested to be endothelium-independent, but still it is the enzymatically produced NO that has been suggested to be involved, making the origin of this NO somewhat unclear (see Aalkjaer & Poston 1996). In the present study, the endothelium of the vessels was left intact, but to reduce the influence of enzymatically derived NO, the experiments were performed in the presence of L-NAME.

The vasodilatory effect of NO is mediated mainly via interaction with soluble guanylyl cyclase (Moncada *et al.* 1991) and also by interaction with potassium channels (Zanzinger *et al.* 1996). The guanylyl cyclase inhibitor ODQ (Garthwaite *et al.* 1995) markedly inhibited the nitrite evoked relaxations of precontracted rat aorta. ODQ has been shown to be a potent guanylyl cyclase inhibitor, without production of superoxide anions (Garthwaite *et al.* 1995) and it prevents NO-mediated smooth muscle relaxation in several vascular beds such as bovine pulmonary artery (Brunner *et al.* 1996), mouse cerebral arterioles (Sobey & Faraci 1997) as well as NO-donor mediated relaxations of rat aortic rings (Olson *et al.* 1997). In the present study, a rightward shift of the nitrite concentration-response curve was evoked and ODQ also suppressed the maximal effect of nitrite by approximately 50%. This fits with earlier reports of ODQ as a mixed competitive and non-competitive antagonist of guanylyl cyclase. The results support the identification of NO as the mediator of nitrite relaxation in the present *in vitro* set-up and that acidic vasodilation via nitrite may involve cGMP. However, it is possible that some NO effects may be related to other mechanisms such as direct interaction with ion channels.

The direct measurement of NO from the artificial organ bath, containing neutral or acidic buffer solution and increasing concentrations of nitrite, is also taken as evidence for an actual NO formation from nitrite, a formation which is increased at low pH. In this set-up, it is also confirmed that the NO production is markedly increased if ascorbic acid is present, independent of pH. Thus, it is clear that ascorbic acid further enhances NO production from acidified nitrite, in line with the

functional experiments. When the smooth muscle relaxatory effects were compared with the amount of NO produced at same nitrite concentration but under different conditions, they were shown to be highly correlated, that is, the amount of NO produced represented approximately the same vascular effect. Furthermore, the vascular effects evoked by SNAP were also highly correlated to its NO production and the NO-dose-effect curve for SNAP was in the same range as the corresponding curve for nitrite. These results also support that NO is the mediator of the nitrite-induced aortic relaxation. One might argue that the slightly different composition of the buffer solutions used in this study in some way affects the sensitivity to NO. If this were true, some of the results in this study would be explained by other things than differences in the amount of NO generated from nitrite at neutral and acidic pH. However, when looking at relaxations evoked by an NO-donor (SNAP), in the two different buffer solutions, we found only minor differences in vasorelaxatory effects (about 10%), to be compared with 30–40% difference between the relaxatory effect evoked by nitrite in neutral and acidic buffer. Thus, this explanation is unlikely.

In conclusion, the present study demonstrates that inorganic nitrite in physiological concentrations evokes vasodilation, most likely through NO release. This NO release and in parallel the vasodilatory effect, is increased if the pH of the environment is reduced to levels normally found in tissues during ischaemia/hypoxia or increased metabolic activity. This suggests that non-enzymatically derived NO contributes to the 'metabolic-acidic' local blood flow regulation, results that have to be confirmed in future *in vivo* studies.

The present study was supported by grants from the Swedish Society of Medicine, the Tore Nilsson Foundation, the Swedish Medical Research Council (12585, 12586, 10354) and funds from the Karolinska Institute. We are grateful for expert technical assistance from Margareta Stensdotter.

REFERENCES

Aalkjaer, C. & Poston, L. 1996. Effects of pH on vascular tension: which are the important mechanisms? *J Vasc Res* **33,** 347–359.

Bartsch, H., Ohshima, H. & Pignatelli, B. 1988. Inhibitors of endogenous nitrosation: mechanisms and implications in human cancer prevention. *Mutat Res* **202,** 307–324.

Bonne, C., Muller, A. & Villain, M. 1998. Free radicals in retinal ischemia. *Gen Pharmacol* **30,** 275–280.

Brunner, F., Schmidt, K., Nielsen, E.B. & Mayer, B. 1996. Novel guanylyl cyclase inhibitor potently inhibits cyclic GMP accumulation in endothelial cells and relaxation of bovine pulmonary artery. *J Pharmacol Exp Ther* **277,** 48–53.

Deshmukh, D.R., Ghole, V.S., Marescau, B. & De Deyn, P.P. 1997. Effect of endotoxemia on plasma and tissue levels of nitric oxide metabolites and guanidino compounds. *Arch Physiol Biochem* **105,** 32–37.

Duncan, C., Dougall, H., Johnston, P. *et al.* 1995. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nature Med* **1,** 546–551.

Ferrari, R., Agnoletti, L., Comini, L. *et al.* 1998. Oxidative stress during myocardial ischaemia and heart failure. *Eur Heart J* **19** (Suppl. B), B2–B11.

Friedberg, M.A., Hinsdale, M.E. & Shibabi, Z.K. 1997. Analysis of nitrate in biological fluids by capillary electrophoresis. *J Chrom* **781,** 491–496.

Franco-Cereceda, A.K., Källner, G. & Lundberg, J.M. 1993. Capsazepine-sensitive release of calcitonin gene-related peptide from C-fibre afferents in guinea-pig heart by low pH and lactic acid. *Eur J Pharmacol* **238,** 311–316.

Furchgott, R.F. & Zawadzki, J.V. 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288,** 373–376.

Furchgott, R.F., Khan, M.T. & Jothianandan, D. 1987. Comparison of endothelium-dependent relaxation and nitric oxide-induced relaxation in rabbit aorta. *Fed Proc The* **46,** 385.

Garthwaite, J., Southam, E., Boulton, C.L., Nielsen, E.B., Schmidt, K. & Mayer, B. 1995. Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol Pharmacol* **48,** 184–188.

Gaskell, W.H. 1880. On the tonicity of the heart and blood vessels. *J Physiol* **3,** 48–75.

Giannella, E., Mochmann, H.-C. & Levi, R. 1997. Ischemic preconditioning prevents the impairment of hypoxic coronary vasodilatation caused by ischemia/reperfusion. *Circ Res* **81,** 415–422.

Gillespie, J.S., Liu, X. & Martin, W. 1989. The effects of L-arginine and N^{G}-monomethyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. *Br J Pharmacol* **98,** 1080–1082.

Guyton, A.C. & Hall, J.E. 1996. *Textbook of Medical Physiology.* W.B. Saunders Company, Philadelphia, PA.

Goodman, L. & Gilman, A. 1941. *The Pharmacological Basis of Therapeutics*, The Macmillan Company, New York.

Högestätt, E.D., Andersson, K.-E. & Edvinsson, L. 1983. Mechanical properties of rat cerebral arteries as studied by a sensitive device for recording of mechanical activity in isolated small blood vessels. *Acta Physiol Scand* **117,** 49–61.

Ignarro, L., Buga, G., Wood, K., Byrns, R. & Chaudhuri, G. 1987. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* **84,** 9265–9269.

Ishizaka, H. & Kuo, L. 1996. Acidosis-induced coronary arteriolar dilation is mediated by ATP-sensitive potassium channels in vascular smooth muscle. *Circ Res* **78,** 50–57.

Jackson, T.S., Xu, A., Vita, J.A. & Keaney, J.F. Jr. 1998. Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circ Res* **83,** 916–922.

Levine, G.N., Frei, B., Koulouris, S.N., Gerhard, M.D., Keaney, J.F. Jr & Vita, J.A. 1996. Ascorbic acid reverses

endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation* **93,** 1107–1113.

Leone, A.M., Francis, P.L., Rhodes, P. & Moncada, S. 1994. A rapid and simple method for the measurement of nitrite and nitrate in plasma by high performance capillary electrophoresis. *Biochem Biophys Res Comm* **200,** 951–957.

Lundberg, J.O.N., Carlsson, S., Engstrand, L., Morcos, E., Wiklund, N.P. & Weitzberg, E. 1997. Urinary nitrite: more than a marker of infection. *Urology* **50,** 189–191.

Lundberg, J.O.N., Weitzberg, E., Lundberg, J.M. & Alving, K. 1994. Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* **35,** 1543–1546.

Mayer, B., Schmidt, K., Humbert, P. & Böhme, E. 1989. Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca^{2+}-dependently converts L-arginine into an activator of soluble guanylyl cyclase. *Biochem Biophys Res Comm* **164,** 678–685.

Moncada, S., Palmer, R.M.J. & Higgs, E.A. 1991. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* **43,** 109–142.

Nathan, C.F. & Hibbs, J.B. 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr Op Immunol* **3,** 65–70.

Nishiyasu, T., Ueno, H., Nishiyasu, M. *et al.* 1994. Relationship between mean arterial pressure and muscle cell pH during forearm ischaemia after sustained handgrip. *Acta Physiol Scand* **151,** 143–148.

Olson, L.J., Knych, E.T. Jr, Herzig, T.C. & Drewett, J.G. 1997. Selective guanylyl cyclase inhibitor reverses nitric oxide-induced vasorelaxation. *Hypertension* **29,** 254–261.

Palmer, R.M.J., Ferrige, A.G. & Moncada, S. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327,** 524–526.

Sobey, C.G. & Faraci, F.M. 1997. Effects of a novel inhibitor of guanylyl cyclase on dilator responses of mouse cerebral arterioles. *Stroke* **28,** 837–843.

Victor, R.G., Bertocci, L.A., Pryor, S.L. & Nunnally, R.L. 1988. Sympathetic nerve discharge is coupled to muscle cell pH during exercise in humans. *J Clin Invest* **82,** 1301–1305.

Weitzberg, E. & Lundberg, J.O.N. 1998. Nonenzymatic nitric oxide production in humans. *NO Biol Chem* **2,** 1–7.

You, J.P., Wang, Q., Zhang, W. *et al.* 1994. Hypercapnic vasodilatation in isolated rat basilar arteries is exerted via low pH and does not involve nitric oxide synthase stimulation or cyclic GMP production. *Acta Physiol Scand* **152,** 391–397.

Zanzinger, J., Czachurski, J. & Seller, H. 1996. Role of calcium-dependent K^+ channels in the regulation of arterial and venous tone by nitric oxide in pigs. *PflGers Arch* **432,** 671–677.

Zsotér, T., Banderman, L. & Chappel, C.I. 1961. The effect of 'local' pH changes on blood flow in the dog. *Am Heart J* **61,** 777–782.

Zweier, J.I., Wang, P., Samouilov, A. & Kuppusamy, P. 1995. Enzyme-independent formation of nitric oxide in biological tissues. *Nature Med* **1,** 804–809.